

Enzymatic saccharification of hot-water pretreated corn fiber for production of monosaccharides

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Abstract

Corn fiber, currently produced at wet milling facilities, is readily available as a potential feedstock for production of fermentable sugars. Destarched corn fiber (DSCF) can be conveniently prepared for enzymatic saccharification by treating with liquid hot-water. Treating DSCF with hot-water (HW-DSCF) at 160 °C for 20 min dissolved 58% of the solids and 75% of the xylan. Preparations of hydrolytic enzymes were next used to saccharify the cellulose and xylan. The needed enzymes were prepared from culture supernatants of *Trichoderma reesei* Rut C30 and *Aspergillus niger* NRRL 2001, each grown on HW-DSCF. The harvested cultures were found to have a broad range of carbohydrase activities. The enzyme profiles varied considerably from one another and the preparations were determined to be most effective for saccharifying HW-DSCF when used in combination. Monosaccharide sugar yields obtained using the blended preparations were 74 and 54% of the available arabinose and xylose, respectively. Arabinose and xylose yields were both further increased to 80% by increasing the hot-water pretreatment time to 30 min and adding a commercial preparation of β -glucosidase, which also contained β -xylosidase side-activity.

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1. Introduction

The U.S. annual production of ethanol is 3.4 billion gallons of ethanol (Renewable Fuel Association, 2005), which consumes 1.4 billion bushels of corn (National Corn Growers Association, 2005). Ethanol is manufactured from corn by either the dry grind or wet mill process. Corn fiber is a residue of wet milling that is currently incorporated into corn gluten feed, a low-protein animal feed product. Redirecting this fiber for conversion to ethanol would simultaneously increase the ethanol yield from wet milled corn (by as much as 10%) and increase the value of the corn gluten feed by reducing its fiber content. Enough corn fiber is produced each year in the U.S. to generate an additional 385 million gallons of ethanol per year [1].

Corn fiber contains approximately 70% carbohydrates including cellulose, xylan, and residual starch. The first step for converting corn fiber to ethanol is saccharifying these carbohy-

drates to fermentable monosaccharide sugars. Most research on corn fiber conversion to ethanol has focused on pretreating corn fiber with dilute sulfuric acid. Typically this involves lowering the pH to 1.0–1.3 with sulfuric acid and treating at a temperature of 150–180 °C for anywhere from 2 to 20 min [2–5]. The sugar yields from these studies are generally very high [4–6]. However, from a processing view-point, dilute-acid pretreatment has several shortcomings, including the need for expensive reactors capable of withstanding the combination of low-pH and high-temperature, formation of pretreatment-associated side-products that often stall the subsequent fermentation, and generation of acid-related waste streams (e.g., gypsum) that adds to the cost of waste treatment and complicates downstream processing.

Hot-water pretreatment is an effective alternative to dilute-acid for pretreating herbaceous biomass [7–10], including corn fiber [11–13]. The major advantages of hot-water pretreatment compared to dilute-acid are avoiding the use of mineral acid (with its myriad of disadvantages) and reducing sugar degradation products. The technology is also conveniently integrated into a wet milling operation as demonstrated by Ladisch and co-

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workers [14]. However, corn fiber treated with hot-water requires further processing than that treated with dilute-acid because the former is not severe enough to saccharify xylan sugars, as needed for their fermentation. Fermentation of xylan is critical because it represents approximately 50 wt% of the corn fiber carbohydrates [5]. Recovering corn fiber xylan as monosaccharide sugars is difficult because its xylan is exceedingly complex in structure and resilient to treatment with most commercially available xylanases.

This paper examines the feasibility of using custom hydrolytic enzyme blends for recovering carbohydrates from hot-water treated corn fiber as monosaccharide sugars. Enzymes were prepared by growing *Trichoderma reesei* Rut C30 and *Aspergillus niger* NRRL 2001 on hot-water treated corn fiber. The recovered cultures were profiled for carbohydrase activities and evaluated on hot-water treated corn fiber for release of monosaccharide sugars. The effect of adding additional enzyme activities and modifying the pretreatment conditions was also examined for the possibility of increasing final sugar yields.

2. Materials and methods

2.1. Materials

Microbial strains were obtained from the ARS Culture Collection (NCAUR, Peoria, IL). Corn fiber was received from Aventine Renewable Fuels (Perkin, IL) and stored at -20°C . Feruloyl esterase is a recombinant purified enzyme originating from *Clostridium thermocellum* that has been over expressed in *E. coli* [15]. A purified xylosidase/arabinosidase, isolated from *Selenomonas ruminantium*, was expressed in and purified from *E. coli* [16]. The following commercial enzymes were used: glucoamylase (Optidex L-400, Genencor, Palo Alto, CA), glucoamylase (Megazyme, Wicklow, Ireland), and β -glucosidase (NS50010, Novozymes A/S, Bagsvaerd, Denmark). All other chemicals and media ingredients were of research quality and were purchased from either Fisher Scientific (Hampton, NH) or Sigma Chemicals (St. Louis, MO).

2.2. Corn fiber destarching

A 23% (w/w) corn fiber slurry was prepared in citrate phosphate buffer (pH 4.8, 0.817 g citric acid, and 0.604 g K_2HPO_4 per liter H_2O). Glucoamylase (Megazyme) (3 ml) was added and the mixture was incubated at 50°C for 18 h. The solids were recovered and washed repeatedly with distilled water until the rinse ran clear. The destarched corn fiber (DSCF) was dried at 55°C for 18 h using a convection oven (Fisher Scientific). The material was subsequently ground using a coffee grinder (Braun GmbH, Kronberg, Germany). Material used for hydrolysis assays was ground to pass through a 20 mesh screen. Final xylan and starch concentrations were measured by treating 0.1 g, db DSCF with 2N trifluoroacetic acid (1 ml) for 1 h at 100°C and analyzing for released sugars as described below. Cellulose content was estimated from literature values [5] for corn fiber sampled from the same wet mill.

2.3. Pretreating DSCF with hot-water

DSCF was mixed with distilled water at 2% (w/w) for digestion assays. The DSCF mixture was pretreated using 316 stainless steel pipe reactors (40 ml working volume) with threaded caps, which were placed in a fluidized sand bath (Model 01187-00 bath and 01190-72 temperature controller, Cole-Parmer, Vernon Hills, IL). The mixture was heated to and kept at 160 or 180°C for 0–30 min before being quickly cooled in a water bath. The internal pipe reactor temperature was monitored using a thermocouple probe inserted into one of the pipe reactors.

2.4. *T. reesei* and *A. niger* cultivation

Dry fungal conidia were hydrated and inoculated onto PDA (0.4%, w/v, potato; 2.0%, w/v, dextrose; 2.0%, w/v, agar) solid medium and cultivated at 28°C for 7 days. The inoculation culture was prepared by transferring two loops of the grown mycelia with conidia to shake flasks (250 ml) each filled with 50 ml potato (0.4%, w/v) dextrose (2%, w/v) medium. Flasks were shaken at 250 rpm and incubated at 28°C for 72 h. A 5% (v/v) inoculum was transferred to the production culture, which contained 50 ml of medium in a 250 ml Erlenmeyer flask. The production medium contained per liter: 15 g KH_2PO_4 , 20 g corn steep liquor (Sigma Chemicals), 5.0 g ammonium sulfate, 0.5 g $\text{Mg}(\text{SO}_4)_2 \cdot 7\text{H}_2\text{O}$, 1.0 g Tween 80, and 40 g of either untreated or hot-water treated (160°C , 20 min) DSCF. The basal medium was adjusted to pH 4.8 and autoclaved separately from the DSCF. Fungal cultures were grown at 28°C with agitation (250 rpm) for 8 days in an Innova 4230 Shaker/Incubator (New Brunswick Scientific Co., Inc., Edison, NJ). Supernatants were recovered and concentrated five-fold using Amicon CentriconPlus 80 (Millipore, CA) centrifugal filter devices with a 10 kDa nominal molecular weight limit. Enzyme preparations were stored at -80°C .

2.5. DSCF digestion assays

Untreated or pretreated DSCF (20 g/l), thymol (500 mg/l), and citric acid buffer (pH 4.8, 50 mM) were added to a scintillation vial for a total volume of 4 ml. Next, the appropriate enzyme mixtures were added to the vial. The vials were mixed using a mini tube roller (Belco Glass, Inc., Vineland, NJ) in a constant temperature incubator (Innova 4230). The roller speed was set to 5 and the incubator temperature to 50°C . The reactions were allowed to proceed for 72 h. Digestion reactions were clarified by centrifuging in a micro-centrifuge (13,000 rcf, 10 min, Eppendorf 5415C, Brinkmann Instruments, NY) and the supernatant stored at -20°C until analyzed for total and monomeric carbohydrates. Assays were carried out in duplicate. Factorial experiments were designed and analyzed using Design-Expert version 6.03 (Stat-Ease, Inc., Minneapolis, MN).

2.6. Enzyme assays

Enzyme activities were determined at 50°C and pH 4.8 (50 mM citric acid–NaOH) using published methods. Cellulase was measured as the release of reducing sugars (DNS method) from carboxy-methyl-cellulose (CMC). Xylanase was determined as the release of reducing sugars (DNS method) from soluble oat spelt xylan [17] with one unit defined as the release of 1 μmol sugar/min. Glucoamylase activity was measured by incubating the enzyme preparation with maltose for 15–30 min and measuring released glucose with one unit being defined as release of 1 μmol sugar/min. Xylosidase and cellobiase activities were determined by the release of *p*-nitrophenol from *p*-nitrophenyl glycosides [17] with one unit being defined as one μmole *p*-nitrophenol released/min. Feruloyl esterase activity was determined by measuring conversion of methyl-ferulate to ferulate. Methyl-ferulate was prepared as a concentrated stock (100 mM) in 50% (v/v) DMSO and added to a final concentration of 2 mM. Ferulic acid and methyl-ferulate were measured by reserve phase SpectraSYSTEM liquid chromatography system (Thermo Electron Corporation, Waltham, MA) using an Inertsil C18 column (5 μm , ODS3, PN 0396–250 \times 046, Varian, Torrance CA) combined with a UV2000 ultraviolet detector (310 nm; Thermo Finnigan). Samples were run at ambient temperature and eluted at 0.8 ml/min with a linear gradient from 5 to 50% acidified methanol (containing 0.25% acetic acid) run over 15 min. Reported activities and concentrations are the average of duplicate numbers. Crude protein concentrations were measured using the method of Bradford.

2.7. Analysis of soluble carbohydrates

Total soluble carbohydrates were analyzed by HPLC after being hydrolyzed by treating with 2N TFA for 1 h at 100°C , as previously described [4]. Samples were analyzed for sugars and acids using a SpectraSYSTEM liquid chromatography system equipped with a RI-150 a refractive index detector (Thermo Electron Corporation) and with an organic acids column (Aminex HPX-87H Column, 300 mm \times 7.8 mm, Bio-Rad Laboratories, Inc., Hercules, CA) [4].

3. Results

3.1. Pretreating destarched corn fiber with hot-water

The corn fiber used in this study was partially destarched to minimize background glucose originating from the starch; corn fiber contains up to 20% (w/w) residual starch. The corn fiber was destarched by treating with a purified glucoamylase (Megazyme) and repeatedly washing with distilled water. The partially destarched corn fiber (DSCF) was determined to have the following carbohydrate content (per gram DSCF, db): 145 mg arabinose, 286 mg xylose and galactose, and 276 mg glucose of which 76 mg originated from starch. Xylan and starch associated sugars were measured directly by saccharifying DSCF with acid and measuring released sugars by using HPLC. Cellulose content was estimated from an earlier study on similarly produced corn fiber [5].

DSCF was pretreated at 160 and 180 °C for 0–30 min. Either temperature was sufficient to solubilize >60% of the solids, albeit the lower temperature required longer incubation times (Fig. 1A). Analysis of the hydrolyzed carbohy-

drates revealed that much of the solids released originated from xylan. Each temperature dissolved 74–78% of the available xylan carbohydrates (Fig. 1B and C). In contrast, only 20–21% of the glucans were dissolved, presumably the source of this glucose is starch that was not removed by the earlier glucoamylase treatment. As expected, most of the released carbohydrates were oligomers. Free xylan sugar yields were 17% (160 °C) and 37% (180 °C) for xylose and 58% (160 °C) and 56% (180 °C) for arabinose. For DSCF treated at 180 °C, the maximum yields of arabinose and total released arabinan occurred at 5–10 min. In contrast, the yields of other carbohydrates increased throughout the entire reaction time. The decrease in arabinan-related yields is most likely attributed to the rate of break-down of arabinose to furfural exceeding the rate of arabinose formation (Fig. 1C and D). Pentose sugars (arabinose and xylose) break-down to furfural and hexose sugars (glucose and galactose) to hydroxymethylfurfural (HMF). Furfural concentrations were >400% higher for samples pretreated at the higher temperature. As furfural is a potent inhibitor of fermentation, further experimental work was carried at 160 °C.

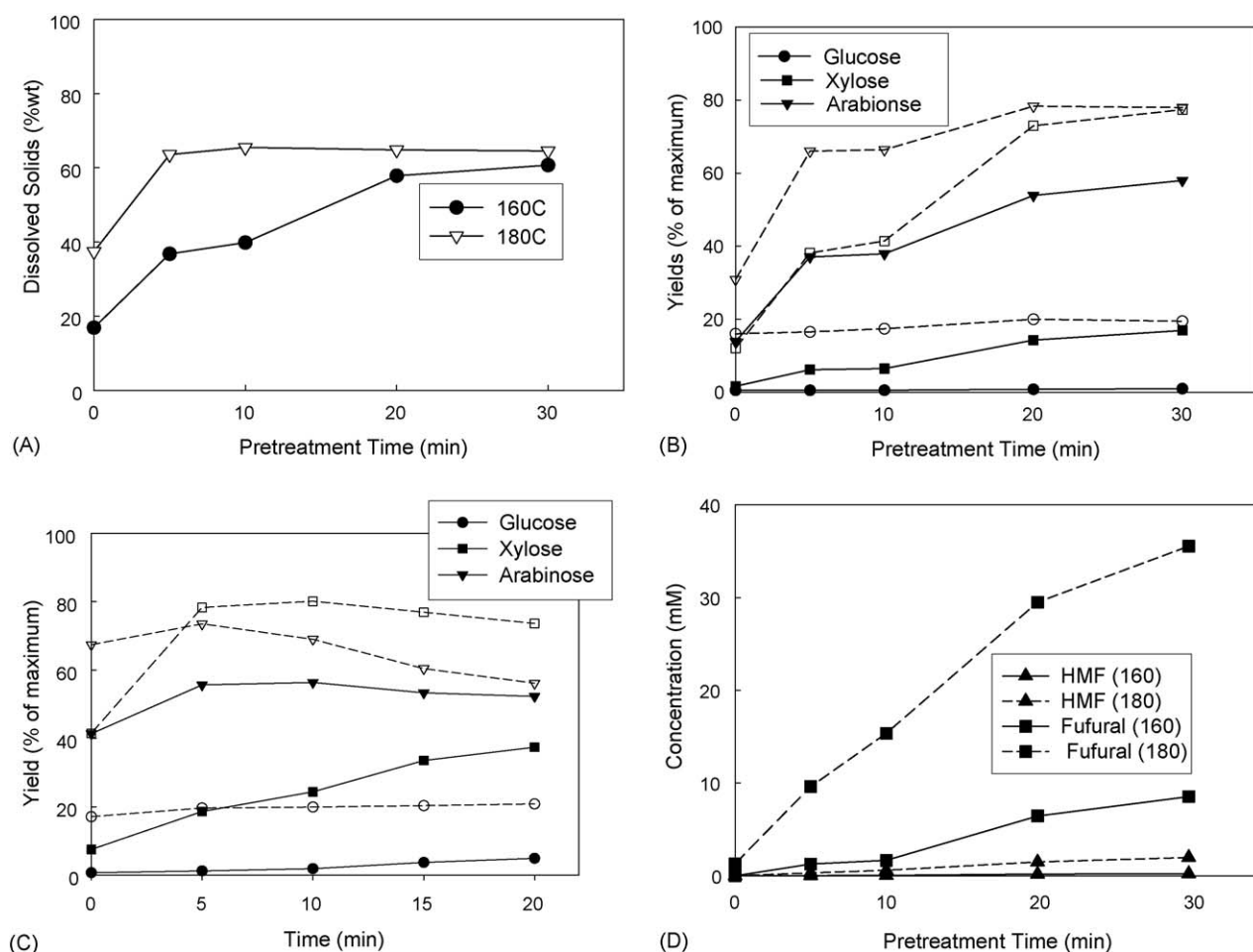


Fig. 1. (A) Solids released from pretreating DSCF in water (2%, w/w, solids) at 160 and 180 °C. (B) Kinetics for the release of carbohydrates and monosaccharides from DSCF treated with water at 160 °C for up to 30 min. (---) Total dissolved carbohydrates and (—) free sugars. (C) Kinetics for the release of carbohydrates and monosaccharides from DSCF treated with water at 180 °C. (---) Total dissolved carbohydrates and (—) free sugars. (D) Production of furfural and HMF during hot-water pretreatment of DSCF at 160 °C (—) and 180 °C (---).

Table 1
Enzyme activity profiles

Enzyme preparation ^a	DSCF pretreatment	Protein (mg/ml)	Activities of enzyme preparations			Comparison of Act. (HW:untreated)		
			Xylanase (U/ml)	Cellulase (U/ml)	FE activity (μM/(min ml))	Xylanase % improvement ^b	Cellulase % improvement ^b	FAE activity % improvement ^b
<i>A. niger</i> 2001	HW	1.24	113.5	5	2.25	57.2	2.8	96.1%
<i>A. niger</i> 2001	Untreated	0.49	72.2	4.8	1.15			
<i>A. niger</i> 2270	HW	1.27	103.3	6	2.20	151.2	28.7	1613.1%
<i>A. niger</i> 2270	Untreated	0.42	41.1	4.7	0.13			
<i>T. reesei</i> RUT C30	HW	1.35	64.4	7	nd ^c	39.1	−15.4	na ^d
<i>T. reesei</i> RUT C30	Untreated	1.42	46.3	8.3	nd			

^a Fungal enzyme mixtures were either prepared from cultures of *A. niger* 2001 or 2270 or *T. reesei* RUT30 grown on HW- or untreated DSCF as a carbon source.

^b % ratio of activities for cultures grown on HW-DSCF vs. untreated DSCF.

^c Not detected.

^d Not applicable.

3.2. Production of hydrolytic enzymes using untreated and pretreated destarched corn fiber

Enzyme activities were compared for cultures of *T. reesei* Rut C30 and *A. niger* strains 2001 and 2270 grown on hot-water treated and untreated DSCF (Table 1). Both *A. niger* strains had higher xylanase activities than the *T. reesei* strain and vice versa for cellulase activities. Interestingly, no feruloyl esterase (FAE) activity was detected in the *T. reesei* preparations. With the exception of cellulase production by *T. reesei* cultures, fungi grown on HW-DSCF produced higher activities and more crude protein than those grown on untreated DSCF (Table 1). The increase was most dramatic for *A. niger* 2001. Based upon these results, preparations from *A. niger* 2001 and *T. reesei* RUT C30 cultured on HW-DSCF were selected for further study.

3.3. Enzymatic digestion of pretreated and untreated DSCF

In a prior study, we investigated the effectiveness of *T. reesei* cultured on DSCF for digesting DSCF and isolated corn fiber xylan [18]. Here, we extend the study by examining the effects of adding enzymes produced by *A. niger* together with those of *T. reesei*. First, the *T. reesei* prep was tested at different loadings on untreated DSCF to find the maximum possible sugar yields. Next, a 1.2 mg/g HW-DSCF, db equal mixture of the *T. reesei* and *A. niger* preparations were evaluated on the same substrate (Fig. 2A). Adding enzymes from *A. niger* to the *T. reesei* preparation increased the combined arabinose and xylose yield from 6.7 to 8.7% of available xylan. Conversely, when *A. niger* alone was added to HW-DSCF, the xylose and arabinose yield was only 4.5%. Therefore, the two enzyme blends complimented each other for releasing sugars from corn fiber.

Still monosaccharides recovered using the enzyme mixture on untreated DSCF corresponded to complete hydrolysis of less than 10% of the added xylan. When DSCF was pretreated by incubating it in hot-water (160 °C) for 20 min (see Section 3.1),

there was a seven-fold increase in the final yield of recovered xylose and arabinose following enzymatic treatment (Fig. 2B). An analogous experiment was conducted using the HW-DSCF as described in the prior paragraph for untreated DSCF and a similar trend was observed: an equal protein mixture of the two enzyme preparations (1.2 mg protein/HW-DSCF, db) released more xylose and arabinose (61%) than either enzyme preparation by itself (*T. reesei* = 44% and *A. niger* = 49% each at 0.6 mg protein/HW-DSCF, db) (Fig. 2B). Blending the enzyme preparations also gave higher glucose yields than adding them separately (Fig. 2C). While the *T. reesei* preparation had higher cellulase activity than the *A. niger* preparation (Table 1), the *A. niger* preparation contained higher auxiliary enzyme activities (e.g., xylanase and feruloyl esterase) and these later activities may explain the higher glucose yield resulting from addition of *A. niger* enzymes.

In addition to monomeric sugars, total released xylan was measured by completely digesting the solid-free syrup with acid prior to measuring sugars by HPLC. Total dissolved xylan-related carbohydrates mirrored that of the free sugars (data not shown). Treating with hot-water alone dissolved 73% of the xylan carbohydrates. A combination of the two enzymes (0.6 mg protein/g HW-DSCF loading of each) dissolved 86% of the xylan, while adding either *A. niger* alone (79% at 0.6 mg protein/g HW-DSCF loading) or *T. reesei* alone (75% at 0.6 mg protein/g HW-DSCF loading) gave lower yields. Based upon their improved effectiveness in combination, an equal mixture of the two enzyme preparations was used for subsequent experiments.

3.4. Effect of adding auxiliary enzyme preparations to pretreated DSCF

Further work was directed towards improving the xylose sugar yield by optimizing the loading of the *A. niger*/*T. reesei* enzyme mixture and by supplementing it with additional

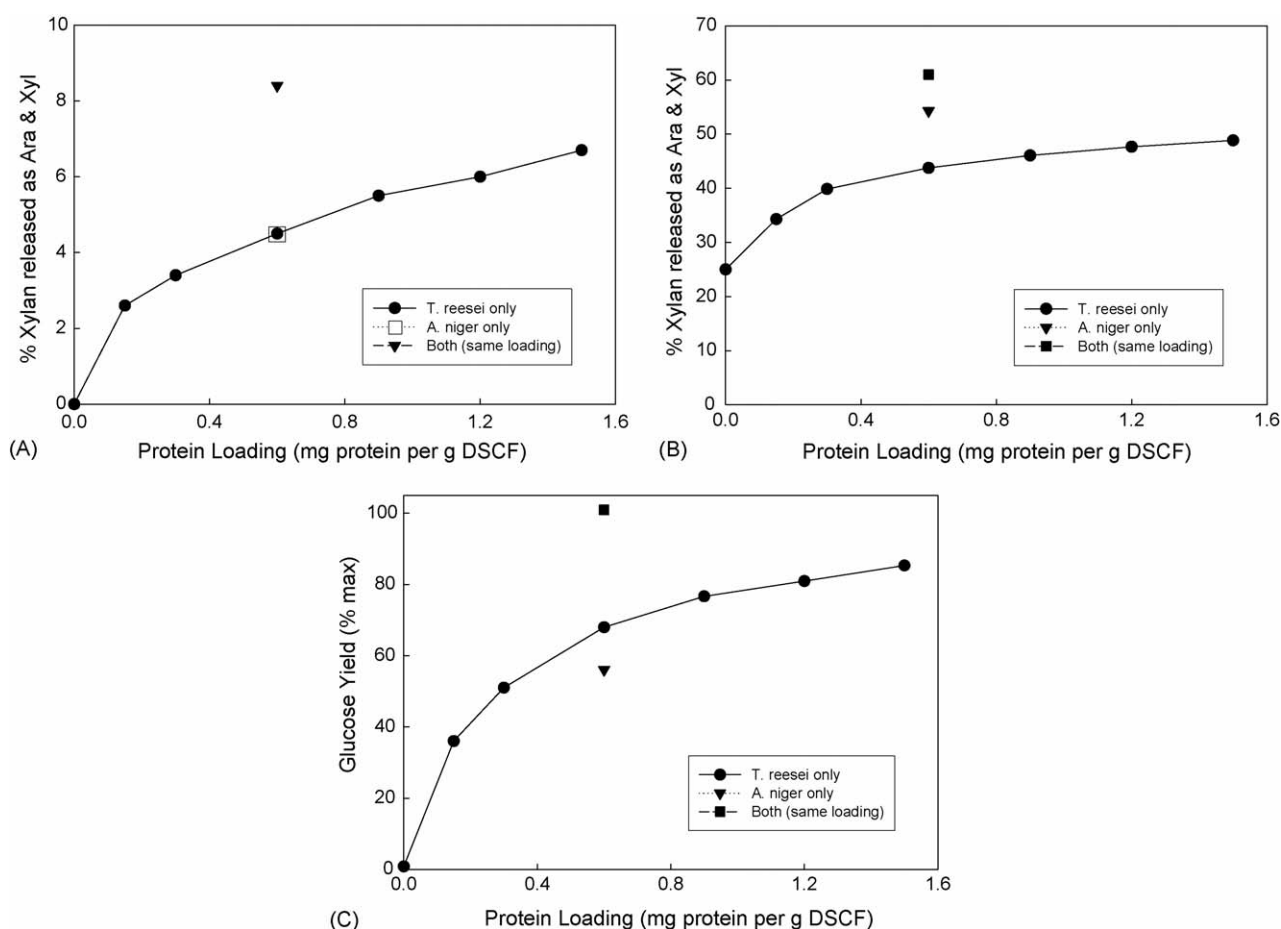


Fig. 2. (A) Xylan associated sugar yields from saccharifying DSCF using varying loadings of *T. reesei* enzyme preparation and the affect of combining *T. reesei* and *A. niger* enzyme preparations. (B) Xylan associated sugar yields from saccharifying hot-water pretreated DSCF hydrolysate (20 min, 160 °C) using varying loadings of *T. reesei* enzyme preparation and the affect of combining *T. reesei* and *A. niger* enzyme preparations. (C) Glucose yields from saccharifying hot-water pretreated DSCF hydrolysate (20 min, 160 °C) using varying loadings of *T. reesei* enzyme preparation and the affect of combining *T. reesei* and *A. niger* enzyme preparations.

enzymes. In this series of experiments, HW-DSCF was treated with different amounts of the *A. niger*/*T. reesei* enzyme mixture and with three auxiliary enzymes (Table 2). The *A. niger*/*T. reesei* mixture gave quantitative glucan conversion and sugar yields of arabinose and xylose of 72 and 54%, respectively (Fig. 3). Furthermore, only a small increase in yields was observed when the loading was increased from 1.2 to 1.8 mg/g, indicating that the digestion was not limited by enzyme concentration beyond a 1.2 mg/g loading. Essentially all of the xylan was dissolved at a loading of 0.90 mg/g and above (data not shown). Possible explanations for the enzyme mixtures' inability to release all of

the arabinose and xylose from xylan include the absence of necessary enzyme activities for removing some of the side-groups and insufficient exposure of hydrolysis sites for enzyme action by the pretreatment.

These possibilities were explored by supplementing the *A. niger* and *T. reesei* enzyme mixture with additional enzymes and increasing the pretreatment time from 20 to 30 min. The additional enzymes used for this experiment were a purified β -xylosidase, a purified FAE, and a commercial mixture of glucan-related enzymes (cellobiase and glucoamylase; Table 2). FAE was included as a treatment because ferulic acid forms an

Table 2
Enzyme activities^a (all activities in U/ml of enzyme preparation)

Enzyme preparation	Assay pH	Protein (mg/ml)	Xylanase	Cellulase	Glucoamylase	FAE	β -Glucosidase	β -Xylosidase
<i>A. niger</i> 2001	4.8	1.2	257	11.3	0.97	2.1	1.73	0.1
<i>T. reesei</i> RUT C30	4.8	1.2	28	13.1	0.54	nd ^b	0.65	6.3
β -Xylosidase	4.8	0.2					nd ^b	818
Feruloyl esterase	4.8	1.3				3.7		
β -Glucosidase ^c	4.8	33.4			1180		263	6.3

^a Glucoamylase from Megazyme had glucoamylase activity of 1470 U/ml and that from Genencor (AM300L) had 4070 U/ml 200 U/ml.

^b Not detected.

^c Novozyme 50010.

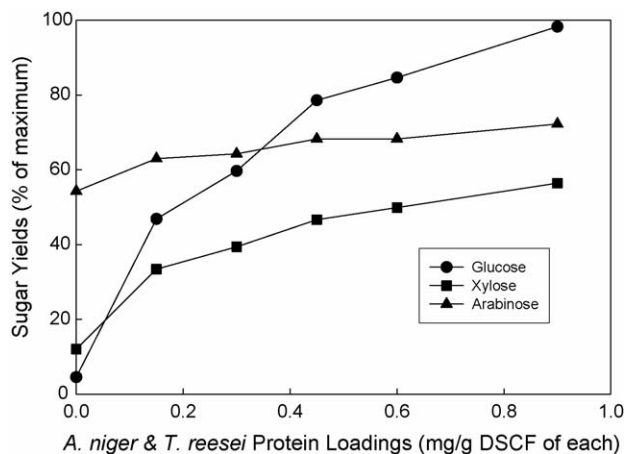


Fig. 3. Sugar yields from saccharifying hot-water pretreated DSCF hydrolysate (20 min, 160 °C) using varying loadings of an equal protein mixture of *T. reesei* and *A. niger* enzyme preparations.

ester linkage with arabinose. To capture interactions, these four factors were evaluated using a full factorial experimental design with two replicates per trial. The measured responses were yields of arabinose, glucose, and xylose. Each factor was analyzed for significance (by ANOVA) and significant factors ($P < 0.05$) fitted by linear regression. The predicted yields (from the linear fit of the data) for different treatments are shown in Fig. 4A (correlation values noted in figure caption). The base treatment was defined as 20 min cooking time followed by saccharification with a 0.6 mg/g loading of each *A. niger* and *T. reesei* preparations. Sugar yields were improved relative to the base case by increasing the cook time to 30 min ($P < 0.0001$), adding the glucan hydrolyzing enzymes ($P < 0.0001$), and (for arabinose) by supplementing with FAE ($P < 0.0001$); adding β -xylosidase had no effect. Improvements in sugar yields from these treatments were 1.7–14% for recovery of arabinose, glucose, and xylose. Also, combined effects were additive (e.g., probability of binary interactions = $P < 0.10$). So, combining the treatments all together led to the highest sugar yields, which were 80% for arabinose, 94% for glucose, and 80% for xylose.

It was unexpected that treating HW-DSCF with a mixture of glucoamylase and β -glucosidase would improve the yields for all of the sugars. Therefore, this result was examined in more detail by conducting an additional factorial experiment. In this experiment, the three factors were: cellobiase, commercial glucoamylase (Genencor, the same enzyme product used previously), and a purified glucoamylase (Megazyme). The design was a 3×2 factorial, where the glucoamylases were treated as the same treatment with three levels: no glucoamylase, the commercial mixture (Genencor), and the purified enzyme (Megazyme). HW-DSCF pretreated for 30 min was used as the substrate. Once again the measured responses were yields of arabinose, glucose, and xylose, and the results were fitted by linear regression.

The results from the model are shown in Fig. 4B. The base case was HW-DSCF saccharified with just the *A. niger*/*T. reesei* enzyme mixture (0.6 mg of each per gram DSCF, db). Adding β -glucosidase improved all the sugar yields: arabi-

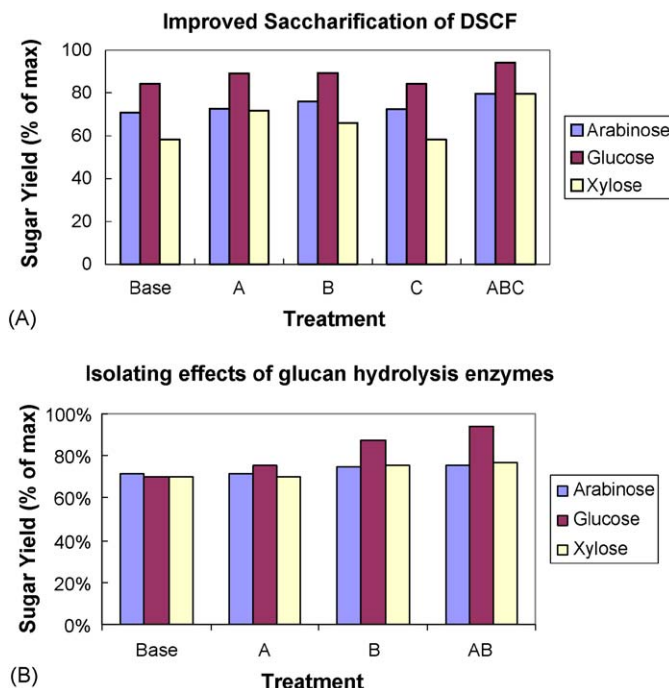


Fig. 4. (A) Improving saccharification of HW-DSCF: (A) base case (treated at 160 °C for 20 min and digested with *A. niger* and *T. reesei* preparations (0.6 mg protein of each per gram DSCF), (B) 30 min pretreatment, (C) addition of glucan hydrolyzing enzymes (100 U β -glucosidase/g DSCF; 40 GAU/g DSCF), (D) addition of feruloyl esterase (3.7 U/g DSCF), and (E) combination of all these treatments. Results are not shown for reactions containing β -xylosidase (100 U/g DSCF) because the addition did not affect yields. See Table 1 and Section 1 for definition of activity units. Yields plotted were calculated from linear regression model fitted to factorial data; regression coefficients were 0.94 (arabinose), 0.79 (glucose), and 0.98 (xylose). (B) Resolving the effect of adding glucoamylase and β -glucosidase on saccharification of HW-DSCF: (Base) base case: (160 °C for 30 min and digested with *A. niger* and *T. reesei* preparations (0.6 mg protein of each per gram DSCF), (B) addition of β -glucosidase (100 U/g DSCF), and (AB) both Genencor glucoamylase and β -glucosidase. Results are not shown for reactions containing purified glucoamylase (25 U/g DSCF) because the addition did not affect yields. See Table 1 and Section 2 for definition of activity units. Yields plotted were calculated from linear regression model fitted to factorial data; regression coefficients were arabinose (1.00), glucose (1.00), and xylose (1.00).

nose by 3.4% ($P < 0.0001$), glucose by 23.7% ($P < 0.0001$), and xylose by 5.6% ($P < 0.0001$). It should be pointed out that the β -glucosidase used for this experiment was a commercial preparation as opposed to a purified enzyme and, therefore, increases in yields may be related to additional (and unreported) activities present in this preparation. In particular, β -xylosidase activity was detected in this enzyme mixture (Table 2). Adding Genencor glucoamylase only improved the yield of glucose by 5.3% ($P < 0.0001$). The effect of treating HW-DSCF simultaneously with β -glucosidase and Genencor glucoamylase was additive, which suggests the two preparations have different substrate targets. Adding Megazyme glucoamylase had no effect on any of the sugar yields. While it may appear surprising that adding the Megazyme glucoamylase did not improve the yield of glucose, it should be noted that the substrate had already been mostly destarched and glucoamylase activity might already have been present in the fungal enzyme blend. The maximum yields

from this experiment were 76% for arabinose, 94% for glucose, and 77% for xylose. Therefore, adding either Genencor glucoamylase or β -glucosidase increased the yields of sugars from HW-DSCF, but adding β -glucosidase had a greater effect.

4. Discussion

Hot-water treatment of corn fiber is an efficient method for dissolving intact xylan. However, these dissolved carbohydrates still need to be saccharified to monosaccharides before they are fermentable by most ethanol producing microorganisms. This is problematic because commercially available xylanases that have been evaluated are largely ineffective for saccharifying corn fiber (pericarp). For example, Hespell et al. [17] was only able to release 18% of the sugars from ammonia fiber explosion (AFEX) pretreated corn fiber xylan using a combination of industrial enzymes, including xylanases, pectinases, and cellulase.

Corn fiber xylan is thought to be resistant to enzymatic digestion because the backbone is highly substituted (80%) with carbohydrate side-groups [17,19]. The high degree of substitution and heterogeneity of these side chain residues (e.g., acetic acid, arabinose, galactose, glucuronic acid, phenolic acids, and xylose [17,20]) suggests that a complex mixture of enzyme activities would be required to completely digest xylan to monomeric sugars and acids [17,21]. The substituted phenolic acids largely consist of ferulic acid monomers (2.61 wt%) and dimers (1.33–2.5 wt%) [19,22]. Simple feruloyl acid groups block most commercial xylanase because they do not contain FAE activity [23]. The diferulates further impede the activity of the enzymes by forming chemical bridges interconnecting xylan chains. These bonds are particularly troublesome because they are resistant to direct enzymatic cleavage [23].

In the absence of effective commercial enzyme blends, custom blends were produced by culturing *A. niger* and *T. reesei* on DSCF. DSCF was chosen as the substrate because earlier work showed that *T. reesei* cultures grown using this carbon source had higher xylanase activities versus those grown using lactose [18]. In the first experiment, fungi were grown on untreated and hot-water pretreated DSCF and each culture measured for enzyme activities. We started with a comparison of HW and untreated DSCF because it was suspected that the fungi might not be able to efficiently digest the native xylan (see above discussion). The results confirmed our suspicions because the fungi cultured on HW-DSCF produced higher enzyme activities than those grown on untreated DSCF (Table 1).

The enzyme profiles for *T. reesei* Rut C30 and *A. niger* 2001 show these two strains produce very different ratios of enzyme activities (Table 2). In particular, unlike *A. niger*, *T. reesei* Rut C30 produced no FAE. Therefore, it was thought that supplementing *T. reesei* cellulases with *A. niger* enzymes might result in a more effective enzyme mixture because the two enzyme preparations would complement each other. In fact, the combined preparations did give higher sugar yields than either enzyme preparation alone (Fig. 2A and B). However, even when HW-DSCF was treated with larger doses of the blended enzymes, only 54% of the xylose was recovered (Fig. 3).

Two strategies were pursued for further improving sugar yields from xylan: increasing the severity of the pretreatment and providing additional enzymes. Increasing the pretreatment by from 20 to 30 min, was enough to increase the xylose yield by 14% (Fig. 4A and B). Further increases in yield were gained by adding β -glucosidase and commercial glucoamylase (the effects were additive). The maximum monosaccharides released were 80% of the available arabinose and xylose and 100% of the glucose.

It is interesting that increasing the duration of the pretreatment from 20 to 30 min had such a large influence on xylose yields because the disappearances of insoluble xylan and total solids had stopped increasing after 20 min. Perhaps, the longer treatment time increased the digestibility of the xylan by removing non-carbohydrate side-groups and/or further opening up the structure. It was not unexpected that adding β -glucosidase would also increase yields because the fungal enzyme preparations were low on this activity (Table 2) and β -glucosidase is typically added to *T. reesei* enzyme preparations to improve its performance on cellulose containing substrates. Another interesting finding from these experiments is that adding additional FAE improved the arabinose yield, which indicates that some ferulate remains bond to arabinose following hot-water treatment.

Monosaccharide yields reported here (80% of the available arabinose and xylose) are superior to previously reported yields for corn fiber that relied on commercial enzyme products. Typical recoveries reported for xylose from corn fiber were only 0–23% where corn fiber was pretreated either with AFEX, alkaline (pH 12.6, 100 °C, 1 h), or hot-water (121 or 160 °C for 1 h) [6,17,24]. Improved yields of monosaccharides have been reported by others using custom enzyme blends on pretreated corn fiber. In these studies, the yields were 30–67% for recovery of xylose; the stains used to produce the enzymes included: *A. niger* I-1472, *Aureobasidium* sp. strain NRRL Y-2311-1, and *Thermobifida fusca* [24–26]. These yields are still much lower than reported here for the *A. niger*/*T. reesei* blend. While the 80% yield is a promising result for developing hot-water as a practical pretreatment for corn fiber, further work is now required for increasing the concentration of the sugar streams and demonstrating that the produced sugars are readily fermented into ethanol.

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